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(54) Title: METHODS FOR GENETICALLY MODIFYING HEMATOPOIETIC STEM CELLS

(57) Abstract

The invention provides methods of genetically modifying hematopoietic stem cells using viral vectors bearing the vesicular stomatitis virus G-glycoprotein.

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METHOD FOR GENETICALLY MODIFYING HEMATOPOIETIC STEM CELLS

INTRODUCTION

Technical Field

The field of this invention is methods for genetic modification of hematopoietic stem cells.

Background

Mammalian hematopoietic cells provide a diverse range of physiological activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineages, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

All publications cited herein are hereby incorporated herein by reference in their entirety.

Despite the diversity of the nature, morphology, characteristics and function of hematopoietic cells, it is presently believed that these cells are derived from a single progenitor population, termed "stem cells." Stem cells are capable of self-regeneration and may become lineage committed progenitors which are dedicated to differentiation and expansion into a specific lineage. As used herein, "stem cells" refers to hematopoietic cells and not stem cells of other cell types. Further, unless indicated otherwise, "stem cells" refers to human hematopoietic stem cells. U.S. Patent No. 5,061,620 describes a substantially homogeneous stem cell

Table 1

Probable Stem Cell Phenotypes

	NK and T cell markers			B cell markers			Myelomonocytic			Other							P-gp Activity
	CD2	CD3	CD8	CD10	CD19	CD20	CD14	CD15	CD16	CD33	CD34	CD38	HLA-DR	C-Kit	Thy	Rho	
FBM	-	-	-	-	-	-	-	-	-	?	+	-	+	+	+	lo	+
ABM	-	-	-	-	-	-	-	-	-	-	+	?	lo/-	+	+	lo	+
AMPB	-	-	-	-	-	-	-	-	-	lo/-?	+	?	lo/-	?	+	lo	+

The ability of stem cells to undergo substantial self-renewal as well as the ability to proliferate and differentiate into all of the hematopoietic lineages makes stem cells the target of choice for a number of gene therapy applications. Successful gene transfer into stem cells should provide long-term repopulation of an individual with the modified cells and their progeny, which will express the desired gene product. By contrast, gene transfer into more mature hematopoietic cells, such as T cells, at best, provides only transient therapeutic benefit. Thus, there have been world-wide efforts toward finding effective methods of genetically modifying stem cells. For reviews of genetic modification of stem cells see Brenner (1993) *J. Hematother.* *2*:7-17; and Moore and Belmont.

Most efforts to genetically modify stem cells have involved the use of retroviral vectors. Other methods such as liposome-mediated gene transfer or adeno-associated viral vectors have also been used. Retroviral vectors have been the primary vehicle due to the generally high rate of gene transfer obtained in experiments with cell lines, and the ability to obtain stable integration of the genetic material, which ensures that the progeny of the modified cell will contain the transferred genetic material. Retroviral vectors and their use in the transfer and expression of foreign genes are reviewed in Gilboa (1988) *Adv. Exp. Med. Biol. 241*:29; Luskey et al. (1990) *Ann. N.Y. Acad. Sci. 612*:398; and Smith (1992) *J. Hematother. 1*:155-166.

Currently used methods of retroviral transduction into human stem cells have a number of practical limitations. One limitation is the extremely low numbers of stem cells present in any tissue. Therefore, in transductions performed with relatively impure populations of cells, the ratio of virus particles to stem cells will be quite low. This limitation is compounded by the relatively low titers generally obtained with most retroviral vectors, typically in the range of 10^5 to 10^6 infectious virions per milliliter.

In addition, primitive stem cells typically are quiescent in culture; retroviral vectors require target cells to be cycling for stable integration of the retroviral DNA. Cytokines may be used to cause stem cells to cycle; however, the effect of various cytokines in driving stem cells to differentiation remains in question. Likewise, the effect of more differentiated cells in culture on the growth or division of stem cells is not well understood.

Many retroviral vectors currently in use are derived from the Moloney murine leukemia virus (MoMLV). In most cases, the viral gag, pol and env sequences are removed from the virus, creating room for insertion of foreign DNA sequences. Genes encoded by the foreign DNA are usually expressed under the control of the strong viral promoter in the LTR. Such a construct can be packed into viral particles efficiently if the gag, pol and env functions are provided in trans by a packaging cell line. Thus, when the vector construct is introduced into the packaging cell, the gag-pol and env proteins produced by the cell, assemble with the vector RNA to produce infectious virions that are secreted into the culture medium. The virus thus produced can infect and integrate into the DNA of the target cell, but does not produce infectious viral particles since it is lacking essential packaging sequences. Most of the packaging cell lines currently in use have been transfected with separate plasmids, each containing one of the necessary coding sequences, so that multiple recombination events are necessary before a replication competent virus can be produced. Alternatively, the packaging cell line harbors an integrated provirus. The provirus has been crippled so that, although it produces all the proteins required to assemble infectious viruses, its own RNA cannot be packaged into virus. Instead, RNA produced from the recombinant virus is packaged. The virus stock released from the packaging cells thus contains only recombinant virus.

The range of host cells that may be infected by a

retrovirus or retroviral vector is determined by the viral envelope protein. The recombinant virus can be used to infect virtually any other cell type recognized by the env protein provided by the packaging cell, resulting in the integration of the viral genome in the transduced cell and the stable production of the foreign gene product. The efficiency of infection is also related to the level of expression of the receptor on the target cell. In general, murine ecotropic env of MoMLV allows infection of rodent cells, whereas amphotropic env allows infection of rodent, avian and some primate cells, including human cells. Amphotropic packaging cell lines for use with MoMLV systems are known in the art and include, but are not limited to, ΨAM, PA12, PA317, and ΨCRIP. Miller et al. (1985) *Mol. Cell. Biol.* 5:431-437; Miller et al. (1986) *Mol. Cell. Biol.* 6:2895-2902; and Danos et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464. Xenotropic vector systems exist which also allow infection of human cells.

The host range of retroviral vectors has been altered by substituting the env protein of the base virus with that of a second virus. The resulting, "pseudotyped", virus has the host range of the virus donating the envelope protein and expressed by the packaging cell line. Recently, the G-glycoprotein from vesicular stomatitis virus (VSV-G) has been substituted for the MoMLV env protein. Burns et al. (1993) *Proc. Natl. Acad. Sci USA* 90:8033-8037; and PCT patent application WO 92/14829. Unlike typical amphotropic env proteins, the VSV-G protein is thought to mediate viral infection by fusing with a phospholipid component of cell membranes rather than by recognition of a cell surface protein. Since infection is not dependent on a specific receptor, VSV-G pseudotyped vectors have a broad host range. Indeed, MoMLV-based retroviral vectors pseudotyped with VSV-G were shown to have a broad host range, infecting a number of cell lines derived from species such as hamster and fish. These types of cell lines are not normally infected by

viruses containing the MoMLV envelope protein.

The mechanism of infection mediated by the VSV-G protein, however, has not been elucidated. It has been reported that VSV-G interacts with a phosphatidylserine component of cell membranes, but that observation has yet to be verified. Schlegal et al. (1983) *Cell 32*:639-646. As a result, it cannot be predicted with any certainty what cells or cell types would be infected by a VSV-G pseudotyped vector, or with what efficiency transduction might occur.

Summary of the Invention

The invention provides improved methods of transducing hematopoietic stem cells with pseudotyped retrovirus vectors containing the VSV-G protein. The methods comprise using the vectors to transduce a population of hematopoietic cells enriched for stem cells.

Brief Description of the Drawings

Figure 1 is a schematic depiction of the plasmid pME-VSV-G.

Figure 2 is a schematic depiction of the plasmid MFG-lac-Z.

Description of the Specific Embodiments

It has now been found that $CD34^+Thy\text{-}1^+$ mobilized peripheral blood (MPB) cells are transduced with surprisingly high efficiency by a VSV-G pseudotyped retroviral vector as compared to $CD34^+$ adult bone marrow (ABM) cells and as compared to the transduction efficiency of a conventional amphotropic vector. The improvement appears to be specific to the vector and cell type; an amphotropic vector containing the same viral genome showed a somewhat better transduction efficiency in $CD34^+$ ABM cells compared to $CD34^+Thy\text{-}1^+$ MPB cells.

While not intending to be bound by any one theory, the improved transduction efficiency described herein appears

to be due either to the more purified stem cell population, represented by $CD34^+Thy\text{-}1^+Lin^-$ cells as compared to $CD34^+$ cells, or due to the tissue cell source. Stem cells that have been mobilized into the peripheral blood by chemotherapy and/or cytokines may undergo changes in membrane characteristics as a result of the activation or mobilization; these changes may affect binding by VSV-G. Therefore, less highly purified stem cells obtained from MPB may also be suitable for use herein. Alternatively, more primitive hematopoietic cells represented by $CD34^+Thy\text{-}1^+Lin^-$ cells may be preferentially infected by the pseudotyped vector regardless of tissue source. In addition, primitive cells may cycle more when purified from more mature progenitor cells, thereby increasing integration of the retroviral genome. In that case, it is likely that any retroviral vector will transduce pluripotent stem cells more efficiently when a purer stem cell population is transduced as compared to the more heterogenous $CD34^+$ cell population. In the presence of less primitive cells, stem cells will cycle less causing transduction frequency to represent disproportionate transduction of progenitor cells at the expense of stem cells.

As used herein, "stem cells" refers to a population of hematopoietic cells more highly enriched in pluripotent stem cells than the population characterized solely by CD34 expression. In particular, "stem cells" refers to a subpopulation of $CD34^+$ cells having all of the long-term engrafting potential *in vivo*. Animal models for long-term engrafting potential include the SCID-hu bone model and the *in utero* sheep model. For review, see Srour et al. (1992) *J. Hematother.* *1*:143-153 and the references cited therein. In assays for long-term culture-initiating cells (LTCIC), the highly enriched stem cell population will typically have a LTCIC frequency in the range of 1/20 to 1/100; preferably it will have a frequency of at least 1/50. Stem cells are exemplified by $CD34^+Thy\text{-}1^+$ MPB cells and described more fully

in the examples provided herein. Other markers that have been reported to subdivide $CD34^+$ cells, further enriching for stem cells, include, but are not limited to, $CD38^-$; $rhodamine^{lo}$; c-kit $receptor^+$; $HLADR^{lo/-}$; $CD71^-$; and $CD45RA^-$. See Table 1. In fetal tissues and umbilical cord blood, stem cells are highly enriched in the $CD34^{hi}Lin^-$ population as described by DiGiusto et al. (1993) *Blood 84*:421-432.

The highly enriched populations of stem cells may be transduced immediately after purification or maintained in long-term cultures and expanded in number in appropriate media, optionally in conjunction with hematopoietic factors such as LIF, stem cell factor, IL3, IL6, IL7, IL11, GCSF, GMCSF, EPO, MIP-1α and IFNγ, under otherwise conventional conditions.

Genetic modification of the stem cells can be accomplished at any point during their maintenance by transduction with a recombinant DNA construct packaged in a virion containing the VSV-G protein. The resulting transduced cells may then be grown under conditions similar to those for unmodified stem cells, whereby the modified stem cells may be expanded and used for a variety of purposes.

Stem cells may be isolated from any known source of stem cells, including, but not limited to, bone marrow, both adult and fetal, mobilized peripheral blood (MPB) and umbilical cord blood. Initially, bone marrow cells may be obtained from a source of bone marrow, including but not limited to, ilium (e.g. from the hip bone via the iliac crest), tibia, femora, spine, or other bone cavities. Other sources of stem cells include, but are not limited to, embryonic yolk sac, fetal liver, and fetal spleen.

For isolation of bone marrow, an appropriate solution may be used to flush the bone, including, but not limited to, salt solution, conveniently supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient

buffers include, but are not limited to, HEPES, phosphate buffers and lactate buffers. Otherwise bone marrow may be aspirated from the bone in accordance with conventional techniques.

Methods for mobilizing stem cells into the peripheral blood are known in the art and generally involve treatment with chemotherapeutic drugs, cytokines (e.g. GM-CSF, G-CSF or IL3), or combinations thereof. Typically, apheresis for total white cells begins when the total white cell count reaches 500-2000 cells/µl and the platelet count reaches 50,000/µl.

Various techniques may be employed to separate the cells by initially removing cells of dedicated lineage ("lineage-specific" cells). Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies may be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain "relatively crude" separations. Such separations are where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker may remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

The use of separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342). Procedures for separation may include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity

chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique. Techniques providing accurate separation include, but are not limited to, FACS, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

A large proportion of the differentiated cells may be removed by initially using a relatively crude separation, where major cell population lineages of the hematopoietic system, such as lymphocytic and myelomonocytic, are removed, as well as minor populations, such as megakaryocytic, mast cells, eosinophils and basophils. Usually, at least about 70 to 90 percent of the hematopoietic cells will be removed. If desired, a prior separation may be employed to remove erythrocytes, by employing Ficoll-Hypaque separation.

The gross separation may be achieved using methods known in the art including, but not limited to, magnetic beads, cytotoxic agents, affinity chromatography or panning. Antibodies which find use include antibodies to lineage specific markers which allow for removal of most, if not all, mature cells, while being absent on stem cells.

Concomitantly or subsequent to a gross separation, which provides for positive selection, a negative selection may be carried out, where antibodies to lineage-specific markers present on dedicated cells are employed. For the most part, these markers include, but are not limited to, $CD2^-$, $CD3^-$, $CD7^-$, $CD8^-$, $CD10^-$, $CD14^-$, $CD15^-$, $CD16^-$, $CD19^-$, $CD20^-$, $CD33^-$ and glycophorin A; preferably including, but not limited to, at least $CD2^-$, $CD14^-$, $CD15^-$, $CD16^-$, $CD19^-$ and glycophorin A; and normally including at least $CD14^-$ and $CD15^-$. See Table 1. As used herein, Lin^- refers to a cell population lacking at least one lineage-specific marker. The

hematopoietic cell composition substantially depleted of dedicated cells may then be further separated using a marker for Thy-1, whereby a substantially homogeneous stem cell population is achieved. Exemplary of this stem cell population is a population which is $CD34^+Thy\text{-}1^+Lin^-$, which provides an enriched stem cell composition.

The purified stem cells have low side scatter and low to medium forward scatter profiles by FACS analysis. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes. Cells may be selected based on light-scatter properties as well as their expression of various cell surface antigens.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a coarse separation, followed by a fine separation, with positive selection of a marker associated with stem cells and negative selection for markers associated with lineage committed cells.

Compositions highly enriched in stem cells may be achieved in this manner. The desired stem cells are exemplified by a population with the $CD34^+Thy\text{-}1^+Lin^-$ phenotype and being able to provide for cell regeneration and development of members of all of the various hematopoietic lineages.

It should be noted that negative selection lineage selection for lineage specific markers provide a greater enrichment in stem cells obtained from bone marrow than from MPB. The majority of $CD34_+$ cells that are mobilized into the peripheral blood do not express lineage-specific markers and, therefore, Lin_- selection does not significantly enrich over $CD34_+$ selection in the peripheral blood as it does in bone marrow. Selection for $Thy\text{-}1_+$ does enrich for stem cells in both mobilized peripheral blood and bone marrow.

A stem cell composition is characterized by being

able to be maintained in culture for extended periods of time, being capable of selection and transfer to secondary and higher order cultures, and being capable of differentiating into the various lymphocytic and myelomonocytic lineages, particularly B and T lymphocytes, monocytes, macrophages, neutrophils, erythrocytes and the like.

The stem cells may be grown in culture in an appropriate nutrient medium, including, but not limited to, conditioned medium, a co-culture with an appropriate stromal cell line, adhesion molecules, or a medium comprising a synthetic combination of growth factors which are sufficient to maintain the growth of hematopoietic cells.

For conditioned media or co-cultures, various stromal cell lines may be used. Since human stromal cell lines are not required, other stromal cell lines may be employed, including but not limited to rodentiae, particularly murine. Suitable murine stromal cell lines include AC3 and AC6, which are described in Whitlock et al. (1987) Cell 48:1009-1021. Other stromal cell lines may be developed, if desired. Preferably, the stromal cell line used is a passage of AC6, AC6.21 (otherwise referred to as SyS1).

Various devices exist for co-culture of stem cells with stromal cells which allow for growth and maintenance of stem cells. These include devices employing mechanisms including, but not limited to, crossed threads, membranes and controlled medium flow. These may be employed for the growth of the cells for removal of waste products, and replenishment of the various factors associated with cell growth.

Conveniently, tissue culture plates or flasks may be employed where confluent stromal cell layers may be maintained for extended periods of time without passage, but with changing of the tissue culture medium about every five to seven days.

The stem cells may be grown in co-culture by

placing the stem cells onto the stromal cell lines, either directly or separated by a porous membrane. For example, about 3 x 10^4 to 3 x 10^5 cells/ml are placed on a confluent stromal cell layer. The media employed in the co-culture may be any convenient growth medium, including, but not limited to, RPMI-1640 and IMDM either individually or in combination, where appropriate antibiotics to prevent bacterial growth, e.g. penicillin, streptomycin (pen/strep) and other additives, such as pyruvate (0.1-5 mM), glutamine (0.5-5 mM), 2-mercaptoethanol (1-10 x 10^{-5} M, 2-ME) and from about 5-15%, preferably about 10% of serum, *e.g.* FCS. Cytokines may also be added, including, but not limited to, LIF, interleukins, colony stimulating factors, steel factor. Of particular interest are LIF, steel factor, IL-3, IL-6, GM-CSF, G-CSF and MIP-1α.

The factors which are employed may be naturally occurring or synthetic, *e.g.* prepared recombinantly, and may be human or of other species, *e.g.* murine, preferably human. The amount of the factors will generally be in the range of about 1 ng/ml to 100 ng/ml. Generally, for LIF, the concentration will be in the range of about 1 ng/ml to 100 ng/mg, more usually 5 ng/ml to 30 ng/ml; for IL-3, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 100 ng/ml; for IL-6, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml, and for GM-CSF, the concentration will generally be 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml.

In one embodiment, the stem cells are optionally expanded prior to or after transduction. During expansion, the growth factors may be present only during the initial course of the stem cell growth and expansion, usually at least 24 hours, more usually at least about 48 hours to 4 days or may be maintained during the course of the expansion.

For use in clinical settings, it is preferable to transduce the stem cells without prior or subsequent

expansion. In one embodiment therefore, the stem cells are cultured with or without cytokines in an appropriate medium, transduced with the appropriate vector, cultured for approximately 72 hours and reintroduced into the host.

Gene transfer into stem cells may be used to treat a variety of neoplastic, infectious or genetic diseases. For example, one may introduce genes that confer resistance to chemotherapeutic agents, thereby protecting the progeny hematopoietic cells, allowing higher doses of chemotherapy and thereby improving the therapeutic benefit. For instance, the mdr1 gene may be introduced into stem cells to provide increased resistance to a wide variety of drugs including taxol, which are exported by the mdr1 gene product, in combination with the administration of chemotherapeutics such as taxol, e.g. for breast cancer treatment. Similarly, genes that provide increased resistance to alkylating agents, such as melphalan, may be introduced into stem cells in conjunction with high dose chemotherapy. Genes that provide resistance to alkylating agents include, but are not limited to, glutathione-S-transferase and methylpurine DNA glycosylate. Other potential drug-resistance genes that could be introduced into stem cells in combination with high-dose chemotherapy include, but are not limited to: adenosine deaminase (ADA) for resistance to purine analogs; O6-alkylguanine-DNA alkyltransferase for resistance to N-methyl-N-nitrosurea; methylguanine methyl transferase for resistance to nitrosurea BCNU; dihydrofolate reductase for resistance to methotrexate; methylpurine DNA glycosylate for resistance to radiation; and glutacyanase transferase for resistance to platinum analogs.

For viral infections that primarily affect hematolymphoid cells, stem cells may be modified to endow the progeny with resistance to the infectious agent. In the case of human immunodeficiency virus (HIV), for example, specific antisense or ribozyme sequences may be introduced that interfere with viral infection or replication in the target

cells. Alternatively, the introduced gene products may serve as "decoys" by binding essential viral proteins, thereby interfering with the normal viral life cycle and inhibiting replication. Introduction of the apoptosis modulating genes such as bcr-abl into stem cells, may provide resistance to the cell death by apoptosis associated with HIV infection.

Alternatively, stem cells may be modified to produce a product to correct a genetic deficiency, or where the host has acquired a genetic deficiency through a subsequent disease. Genes that may correct a genetic deficiency include, but are not limited to, adenosine deaminase for the treatment of ADA$^-$ severe combined immunodeficiency; glucocerebrosidase for the treatment of Gaucher's disease; beta-globin for the treatment of sickle cell anemia; and Factor VIII or Factor IX for the treatment of hemophilia.

Suitable viral constructs are discussed in Burns et al. (1993) and PCT application no. WO 92/14829 although any functional pseudotype virus containing the VSV-G gene and capable of being packaged by a packaging cell line is suitable for use herein. The viral constructs employed will normally include the VSV-G gene, the foreign gene(s) and a marker gene, which allows for selection of cells into which the DNA has been integrated, as against cells which have not integrated the DNA construct. Various marker genes include, but are not limited to, antibiotic resistance markers, such as resistance to G418 or hygromycin. Less conveniently, negative selection may be used, including, but not limited to, where the marker is the HSV-tk gene, which will make the cells sensitive to agents such as acyclovir and gancyclovir. Alternatively, selections could be accomplished by employment of a stable cell surface marker to select for transgene expressing stem cells by FACS sorting.

The viral constructs can be prepared in a variety of conventional ways. Numerous vectors are now available which provide the desired features, such as long terminal

repeats, marker genes, and restriction sites, which may be further modified by techniques known in the art. The constructs may encode a signal peptide sequence to ensure that genes encoding cell surface or secreted proteins are properly processed post-translationally and expressed on the cell surface if appropriate. Preferably, the foreign gene(s) is under the control of a cell specific promoter as discussed below.

In those instances where a particular polymorphic region of a polymorphic protein, such as a T-cell receptor, major histocompatibility complex antigen, or immunoglobulin subunit is involved with susceptibility to a particular disease, for example an autoimmune disease, the particular exon may be "knocked out" by homologous recombination, so as to provide hematopoietic cells which will not be responsive to the disease.

Expression of the transferred gene can be controlled in a variety of ways depending on the purpose of gene transfer and the desired effect. Thus, the introduced gene may be put under the control of a promoter that will cause the gene to be expressed constitutively, only under specific physiologic conditions, or in particular cell types. Examples of promoters that may be used to cause expression of the introduced sequence in specific cell types include Granzyme A for expression in T-cells and NK cells, the CD34 promoter for expression in stem and progenitor cells, the CD8 promoter for expression in cytotoxic T-cells, and the CD11b promoter for expression in myeloid cells. Inducible promoters may be used for gene expression under certain physiologic conditions. For example, an electrophile response element may be used to induce expression of a chemoresistance gene in response to electrophilic molecules. The therapeutic benefit may be further increased by targeting the gene product to the appropriate cellular location, for example the nucleus, by attaching the appropriate localizing sequences.

In addition, by appropriate use of inducible promoters, expression of various protein products can be achieved at selected levels of differentiation or in selected cell lineages, or even in response to particular chemicals, such as chemoattractants, particular ligands, and the like. Also, as there is better understanding of the manner in which stem cells differentiate to specific lineages, particular lineages, such as megakaryocytes, subsets of T cells, monocytes, and the like can be produced in culture.

Possible methods of transduction include, but are not limited to, direct co-culture of stem cells with producer cells e.g. by the method of Bregni et al. (1992) Blood 80:1418-1422 or culturing with viral supernatant alone, with or without appropriate growth factors and polycations, e.g. by the method of Xu et al. (1994) Exp. Hemat. 22:223-230; and Hughes et al. (1992) J. Clin. Invest. 89:1817.

In many situations, cell immunotherapy involves removal of bone marrow or other source of stem cells from a human host, isolating the stem cells from the source and optionally expanding the stem cells. Meanwhile, the host may be treated to partially, substantially or completely ablate native hematopoietic capability. The isolated stem cells may be modified during this period of time, so as to provide for stem cells having the desired genetic modification. After completion of the treatment of the host, the modified stem cells may then be restored to the host to provide for expression of the foreign gene. The methods of stem cell removal, host ablation and stem cell repopulation are known in the art. If necessary, the process may be repeated to ensure substantial repopulation of the modified stem cells.

To ensure that the stem cells have been successfully modified, a vector-specific probe may be used to verify the presence of the vector in the transduced stem cells or their progeny. In addition, the cells may be grown under various conditions to ensure that they are capable of maturation to all of the hematopoietic lineages while

maintaining the capability, as appropriate, of the introduced DNA. Various tests *in vitro* and *in vivo* may be employed to ensure that the pluripotent capability of the stem cells has been maintained.

The compositions comprising stem cells provide for production of myeloid cells and lymphoid cells in appropriate cultures, cultures providing hydrocortisone for production of myeloid cells (associated with Dexter-type cultures) and B lymphocytes in cultures lacking hydrocortisone, (associated with Whitlock-Witte type cultures). In each of the cultures, mouse or human stromal cells are provided, which may come from various sources, including, but not limited to, AC3, AC6 or stromal cells derived from mouse or human FBM by selection for the ability to maintain stem cells, and the like. Preferably, the stromal cells are AC6.21 and the ability to produce B lymphocytes and myeloid cells is determined in cultures supplied with LIF and IL-6. The stem cells give rise to B cells, T cells and myelomonocytic cells in the *in vivo* assays described below.

To demonstrate differentiation to T cells, fetal thymus is isolated and cultured from 4-7 days at about 25°C, so as to deplete substantially the lymphoid population. The cells to be tested for T cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue may then be transplanted into a scid/scid mouse as described in US Patent No. 5,147,784, particularly transplanting under the kidney capsule.

Specifically, the population of stem cells can be microinjected into HLA mismatched thymus fragments. After 6-10 weeks, assays of the thymus fragments injected with stem cells can be performed and assessed for donor- derived T cells. Injected thymus fragments injected with cells having T cell differentiative capacity will generate and sustain $CD3^+$, $CD4^+$, and $CD8^+$ T cells along with their progenitors.

Further demonstration of the sustained regenerative

ability of stem cell populations may be accomplished by the detection of continued myeloid and B-lymphoid cell production in the SCID-hu bone model. Kyoizumi et al. (1992) Blood 79:1704. To analyze this, one may isolate human fetal bone and transfer a longitudinally sliced portion of this bone into the mammary fat pad of a scid/scid animal. The bone cavity is diminished in endogenous cells by whole body irradiation of the mouse host prior to injection of the test donor population. The HLA of the population which is injected is mismatched with the HLA of the recipient bone cells. Stem cells from human hematopoietic sources sustain B lymphopoiesis and myelopoiesis in the SCID-hu bone model.

For RBCs, one may use conventional techniques to identify BFU-E units, for example methylcellulose culture demonstrating that the cells are capable of developing the erythroid lineage. Metcalf (1977) In: Recent Results in Cancer Research 61. Springer-Verlag, Berlin, pp. 1-227.

A pluripotent stem cell may be defined as follows: (1) gives rise to progeny in all defined hematolymphoid lineages; and (2) limiting numbers of cells are capable of fully reconstituting a seriously immunocompromised human host in all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell by self renewal.

The following examples are provided to illustrate but not limit the invention.

Example 1
Cell sorting and analysis

Apheresed samples were obtained with informed consent from multiple myeloma patients treated at the University of Arkansas Medical Center. The patients were treated on day 1 with cyclophosphamide at 6 g/m^2 (1.5 g/m^2 every 3 hrs x 4 doses). From day 1 until the start of leukopheresis (usually 10-28 days), granulocyte macrophage colony stimulating factor (GM-CSF) was given at 0.25

$mg/m^2/day$. Apheresis for total white cells was started when the peripheral blood white cell count was greater than 500 cells/µl and the platelet count was greater than 50,000 cells/µl. Patients were apheresed daily until from 6×10^8 mononuclear cells (MNC) were collected.

Antibodies to CD2, CD14, CD15, CD16 and CD19 were obtained as FITC conjugates from Becton-Dickinson. Antibody to Thy-1 (GM201) was obtained from Dr. Wolfgang Rettig (Ludwig Institute, New York), and was detected with anti-γ1-PE conjugate from Caltag. Antibody to CD34 (Tük 3) was obtained from Dr. Andreas Ziegler (University of Berlin), and detected with an anti-γ3-Texas Red conjugate (Southern Biotechnologies). The antibody to glycophorin A was obtained as a FITC conjugate from AMAC. The lineage cocktail was a combination of antibodies to CD2, CD14, CD15, CD16, CD19 and glycophorin A.

For cell sorting, fresh MPB samples were elutriated with a JE5.0 Beckman counterflow elutriator equipped with a Sanderson chamber (Beckman, Palo Alto, CA). Cells were resuspended in elutriation medium (Biowhittaker, Walkersville, MD) at pH 7.2, supplemented with 0.5% human serum albumin (HSA). The rotor speed was set at 2000 RPM, the cells were introduced, and the first fraction collected at a flow rate of 9.6 ml/min. Fractions 2 and 3 were collected at the respective flow rates of 14 and 16 ml/min. The larger cells remaining in the chamber were collected after stopping the rotor. Cells were resuspended in RPMI supplemented with 5% HSA, 10 µg/ml DNAse I and penicillin/streptomycin at 50 U/ml and 50 µg/ml, respectively. Fractions 2 and 3 were pooled and incubated with 1 mg/ml heat-inactivated human gamma-globulin to block non-specific Fc binding. Granulocytes were further depleted by incubation with CD15 conjugated to magnetic beads (Dynal M450, Oslo, Norway) followed by magnetic selection.

Anti-CD34 antibody or an IgG3 isotype matched control were added to cells in staining buffer (HBSS, 2% FCS,

10 mM HEPES) for 20 minutes on ice, together with anti-Thy-1 antibody at 5 µg/ml. Cells were washed with a FCS underlay, and then incubated with Texas Red conjugated goat anti-mouse IgG3 antibody and phycoerythrin-conjugated goat anti-mouse IgG1 antibody for 20 minutes on ice. Blocking IgG1 was then added for 10 minutes. After blocking, the FITC-conjugated lineage antibody panel was added, and incubated for another 20 minutes on ice. After a final washing, cells were resuspended in staining buffer containing propidium iodide (PI).

Cells were sorted in the FACSTAR Plus cell sorter equipped with dual argon ion lasers, the primary laser emitting at 488 nm and a dye laser (Rhodamine 6G) emitting at 600 nm (Coherent Innova 90, Santa Cruz, CA). Residual erythrocytes, debris and dead cells were excluded by light scatter gating plus an FL3 (PI) low gate. Following isolation of a cell population by FACS, the sample was diluted 1:1 in HBSS, pelleted, and resuspended in HBSS for hemocytometer counting.

$CD34^+$ cells were positively selected from cadaveric adult bone marrow (ABM) using a biotinylated anti-CD34 antibody (K6.1) and a biotin competition release according to the method described in PCT patent application no. WO94/02016.

Example 2

Production of recombinant vector

The amphotropic virus used was MFG-lacZ. MFG vectors are described in Dranoff et al. (1993) Proc. Natl. Acad. Sci. USA 90:3539-3543. Amphotropic MFG-lacZ was obtained as viral supernatant from Somatix Therapy Corporation (Alameda, California). The viral titer represented in Table 2 was determined by the supplier.

A VSV-G pseudotype virus was prepared using a cDNA clone of the New Jersey isolate of the VSV-G envelope protein

as the source of the VSV-G gene. Rose and Bergmann (1982) Cell 30:753-762. The VSV-G gene was subcloned by polymerase chain reaction (PCR) on a Cetus GeneAmp 9600 machine using 100 ng plasmid; 10 ng each primer and 10 cycles of the following: 30 sec 92°C, 30 sec 55°C, and 1 min 72°C. The following primers were used in PCR:

SEQ ID NO 1 5' GCGCGGGAATTCTGACACTATGAAGTGCCT 3'

SEQ ID NO 2 5' CCGGAGTCTAGAGTGCAGGATTTGAGTTAC 3'

Primer No. 1 was the 5' primer and generated an EcoRI site, Primer No. 2 was the 3' primer and generated an XbaI site. The resulting fragment was digested with EcoRI and XbaI and cloned into plasmid pME-18S digested with EcoRI/XbaI to generate plasmid pME-VSV-G. pME-18S was obtained from DNAX; plasmid pME-VSV-G is depicted in Figure 1. Plasmid MFG-lacZ is depicted in Figure 2.

The pseudotyped virus was prepared by transducing the ANJOU cell line according to the method described by Pear et al. (1993) Proc. Natl. Acad. Sci. USA 90:8392-8396. ANJOU cells used in transfection were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) and grown at 37°C in 5% CO_2. NIH3T3 cells were maintained in similar media but containing 10% (v/v) calf serum instead of FCS.

ANJOU cells were plated at 10^7/15 cm plate 18 hours prior to transfection. Standard calcium phosphate transfection solution (Pear et al. (1993)) was prepared with 30 µg MFG-LacZ and 30 µg pME-VSV-G and added dropwise to cells in media containing 25 µg/ml chloroquine to increase transfection efficiency. Six to 8 hours later the media was changed to 10 ml fresh DMEM, cells were grown an additional 10 hours and the media changed again. Viral supernatants were collected 48 hours after transfection and either (1) used to infect target cells, (2) frozen down at -20°C, or (3) concentrated as discussed below. ANJOU cells were fixed in a 2% paraformaldehyde/0.2% glutaraldehyde solution and

stained with X-Gal to determine the transfection efficiency. Pear et al. (1993). The virus obtained was designated VSV-G-MFG-lacZ.

Concentration and titration of virus

Harvested supernatants from confluent ANJOU producer cells were centrifuged to remove cellular debris at 200 rpm for 10 min, then subjected to ultracentrifugation in a Beckman Model centrifuge in an SW28 rotor at 25,000 rpm at 4°C for 1.5 hours. The pellet was resuspended overnight at 4°C in 300 ml TNE (50 mM Tris-HCl, pH 7.8, 130 mM NaCl, 1 mM EDTA) and then frozen at -20°C. Titered amounts of the concentrated or the non-concentrated virus were used to infect target NIH3T3 cells.

The viruses were titered on target cells plated in 3 ml of appropriate media in 6 cm plates at $2x10^5$ cells per plate to achieve 60-73% confluence at the time of infection. Eighteen to twenty-four hours after seeding, the media was supplemented with 5 µg/ml polybrene and virus was added immediately afterward. Media was changed to 6 ml of fresh media 8 hours after addition of virus to avoid polybrene toxicity. Forty-eight hours after infection, the media was removed and cells were assayed for β-galactosidase activity by X-gal or FACS-Gal according to the method described by Nolan et al. (1988) Proc. Natl. Acad. Sci. USA 85:2603-260 for determination of viral titers. The titration results are shown in column 3 of Table 2.

Transductions were performed as described in Example 3, using concentrated, frozen, virus preparations.

Example 3
Transduction

1 x 10^5 $CD34^+$ (ABM) or 1 x 10^5 $CD34^+Thy^+Lin^-$ (MPB) viable cells obtained as described in Example 1, were suspended in 1 ml of freshly thawed retroviral supernatant with cytokines at the following concentrations: c-kit ligand (#mgen) 100 ng/ml; IL-3 (Sandoz) 25 ng/ml; IL-6 (Sandoz) 50 ng/ml. Protamine sulfate was added at a final concentration of 4 µg/ml. At 24 and 48 hours, supernatant was replaced with freshly thawed retroviral supernatant. Cytokines and protamine sulfate were added at the concentrations listed above. After 72 hours, cells were harvested and transduction frequency was determined.

Alternatively, 1 x 10^5 $CD34^+$ (ABM) or 1 x 10^5 $CD34^+Thy\text{-}1^+Lin^-$ (MPB) cells obtained as described in Example 1 were transduced as described above except that the cells were incubated with the retroviral supernatant for 16 hours without change of supernatant. The results of the 16 hour transduction are shown in Tables 2 and 3.

Example 4
Methylcellulose assay

In order to determine transduction frequency of the stem cells, the following experiment was performed. 5 x 10^3 or 2.5 x 10^3 cells from each transduction were added to 5 ml of methylcellulose (Stem Cell Technologies) containing the following cytokines: c-kit ligand 10 ng/ml; GM-CSF 25 ng/ml; G-CSF 25 ng/ml; IL-3 10 ng/ml; rhEPO 2 units/ml. 1.1 ml of the cell/cytokine methylcellulose mixture was plated onto four 3 cm gridded plates using a 5 ml syringe and 16.5 gauge needle, and the plates were placed in a 37°C incubator for 2 weeks.

After 14 days, 2-5 methylcellulose colonies were pooled into 200 µl PBS for PCR analysis. The cells were vortexed 5 seconds, centrifuged for 30 seconds at 10,000 rpm

in a microcentrifuge, and the PBS was removed by aspiration. 100 µl of Florence Lysing Buffer containing proteinase K (50 mM KCl, 20 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% NP40, 1 mg/ml proteinase K) was added to the cell pellet and vortexed to generate a cell lysate. The cell lysate was incubated 60 minutes in a 60°C water bath and subjected to flash spin. The cells were incubated 15 minutes in a 95°C heat block and subjected to flash spin. The lysates were stored at 4°C.

The lysates were amplified by PCR to determine the presence of the vector in the transduced cells. The PCR assay amplified a 539 bp fragment from MFG-lacZ. A 40 cycle amplification (50 µl total volume) in Perkin-Elmer 480 Cycler using 25 µl lysate and 100 ng each LZ 4927 and LZ 5466 (Keystone) Econopure primers was performed as follows: one cycle 95°C 6 min, 62°C 1 min 72°C 2 min; 4 cycles 95°C 2 min, 62°C 1 min, 72°C 2 min; 35 cycles 95°C 2 min, 62°C 1 min, 72°C 2 min; final extension 72°C 10 min. PCR products were visualized on ethidium bromide agarose gel and the results were verified by Southern Blot hybridization with a lacZ probe. As an alternative to detection by Southern blot hybridization, in some cases, a second, nested amplification was performed using a 30 cycle amplification in PE cycler using 5 µl of primary PCR product and 100 ng each of LZ4953 and LZ5425 (Keystone) Econopure primers.

The results obtained are presented in Table 2. Note that the transduction frequency represented in the final column represents a minimum transduction frequency since it assumes that, in a positive sample, only one colony of each pooled sample was positive. At higher transduction frequencies, the number presented is likely to be an underestimate of the actual transduction frequency.

Table 2
Summary of Transduction
Using Amphotropic and VSV-G Pseudotyped Retroviral Factors

Tissue	Retroviral Vector	Viral Titer	Length of Transduction(hr)	Colonies Pooled/Sample	No. Samples	PCR Positive Samples	Transduction Frequency
$CD34^+Thy^+Lin^-$ (MPB)	MFG-*lacZ*	5-10 x 10^5	72	5	36	10/36	10/180 (6%)
$CD34^+Thy^+Lin^-$ (MPB)	VSV-G-MFG-*lacZ*	2 x 10^6	72	3	20	18/20	$\geq$18/60 ($\geq$30%)
Total $CD34^+$ (ABM)	MFG-*lacZ*	5-10 x 10^5	72	5	36	16/36	$\geq$16/180 ($\geq$9%)
Total $CD34^+$ (ABM)	VSV-G-MFG-*lacZ*	2 x 10^6	72	5	20	11/20	11/100 (11%)
$CD34^+Thy^+Lin^-$ (MPB)	MFG-*lacZ*	5-10 x 10^5	16	5	36	0/36	0/180 (0%)
$CD34^+Thy^+Lin^-$ (MPB)	VSV-G-MFG-*lacZ*	8 x 10^5	16	2	34	3/34	3/68 (4%)
Total $CD34^+$ (ABM)	VSV-G-MFG-*lacZ*	8 x 10^5	16	2	38	1/38	1/76 (1%)

TABLE 3

Gene Transfer Into Hematopoietic Stem/progenitor Cells with VSV-G Pseudotype and Amphotropic Retroviruses: 16hr Transduction

Tissue	Retroviral Vector	Viral Titer	Viable Cell Recovery (per 100K)	MeCell Colony Frequency (Per 10K)	MeCell Transduction Frequency
$CD34^+Thy^+Lin^-$ (MPB)	VSG-G-MFG-lacZ	8×10^5	19K	495 (1/20)	4% (3/68)
$CD34^+Thy^+Lin^-$ (MPB)	MFG-lacZ	5×10^5	51K	577 (1/17)	0% (0/180)
Total $CD34^+$ (ABM)	VSV-G-MFG-lacZ	8×10^5	22K	2430 (1/4)	1% (1/72)

The results presented above show that the VSV-G pseudotyped vector transduces $CD34^+Thy\text{-}1^+Lin^-$ MPB stem cells with three to four fold greater efficiency than $CD34^+$ ABM cells. Transductions were performed for either 72 hours (Table 2) or 16 hours (Tables 2 and 3). While transduction efficiency was higher with the longer transduction, the relative increase in transduction frequency was the same under both conditions. Under both transduction conditions, the VSV-G pseudotyped vector transduced MPB stem cells with much greater efficiency than the comparable amphotropic vector (on the order of five-fold in the 72 hour transduction; the 16 hour transduction cannot be compared since there were no transductions detected with the amphotropic vector). The observed differences are not due to patient variability or variation in the stem cell preparation since, in each case, the MPB samples from a single purification for the same patient were transduced with both the pseudotyped and amphotropic retroviral vectors. By comparison, the amphotropic vector transduced $CD34^+$ ABM cells at a slightly higher frequency than $CD34^+Thy\text{-}1^+Lin^-$ MPB cells, although this difference may not be statistically significant. Even with the amphotropic vector, it is likely that the stem cells are transduced at a higher frequency in the $CD34^+Thy\text{-}1^+Lin^-$ population than in the $CD34^+$ population.

In both the 16 and 72 hour transductions with the VSV-G pseudotyped vector, there appeared to be a greater loss in viable cells than in transductions with the amphotropic vector. The clonogenic potential of the recovered cells in methylcellulose, however, did not appear to be significantly affected. (Table 3) Preliminary observations suggest that much of the loss in viability is attributable to the buffer used in the VSV-G virus preparations, rather than to the VSV-G vector itself.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to

those skilled in the art that certain changes and modifications may be practiced. It should be apparent that one having ordinary skill in this art would be able to surmise equivalents to the invention as described in the claims which follow but which would be within the spirit of the description above. Those equivalents are to be included within the scope of this invention. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

Claims

1. A method for genetically modifying hematopoietic stem cells comprising:

obtaining hematopoietic stem cells; and

transducing the stem cells with a viral vector bearing the vesicular stomatitis G protein.

2. The method according to claim 1 wherein the stem cells are $CD34^{+}Thy\text{-}1^{+}Lin^{-}$.

3. The method according to claim 1 wherein the stem cells are obtained from bone marrow, mobilized peripheral blood or umbilical cord blood.

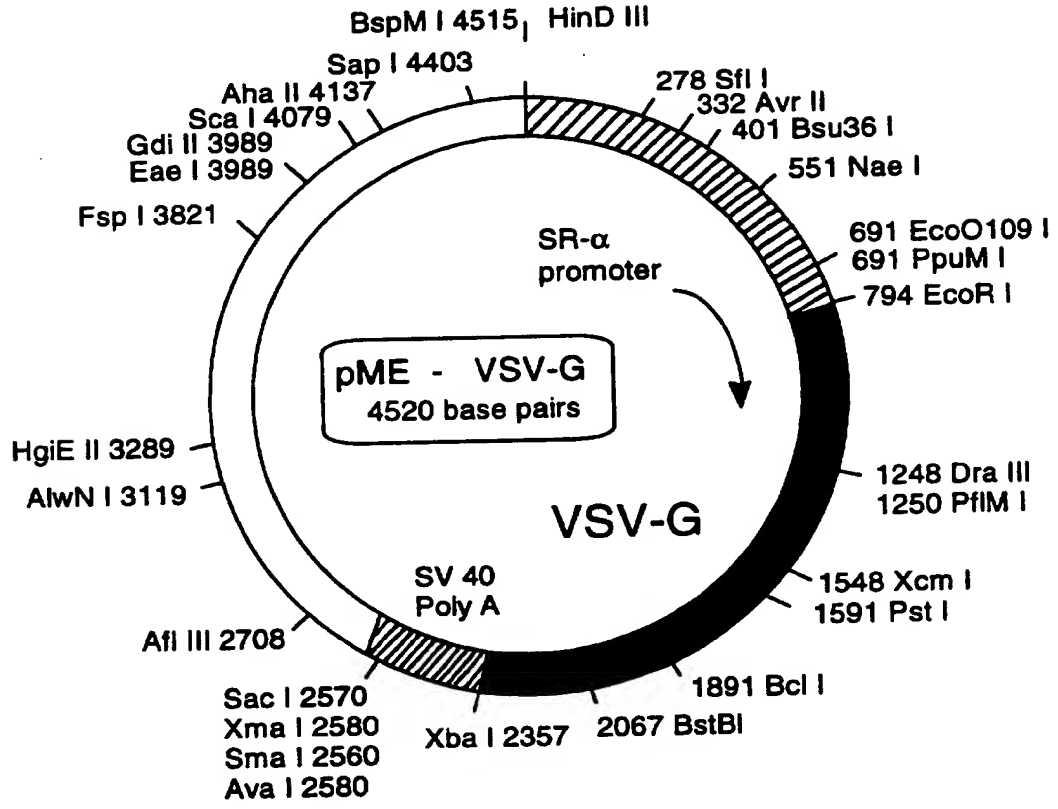
4. The method according to claim 3 wherein the stem cells are obtained from bone marrow or umbilical cord blood and are $CD34^{+}Thy\text{-}1^{+}Lin^{-}$.

5. The method according to claim 3 wherein the stem cells are isolated from mobilized peripheral blood.

6. The method according to claim 5 wherein the stem cells are $CD34^{+}Thy\text{-}1^{+}$.

7. The method according to claim 1 wherein the viral vector is MFG.

8. The method according to claim 1 wherein the stem cells are isolated from mobilized peripheral blood and the viral vector is MFG.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401 Bsu36 I
551 Nae I
691 EcoO109 I
691 PpuM I
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promoter
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1891 Bcl I
2067 BstBI
Xba I 2357
SV 40
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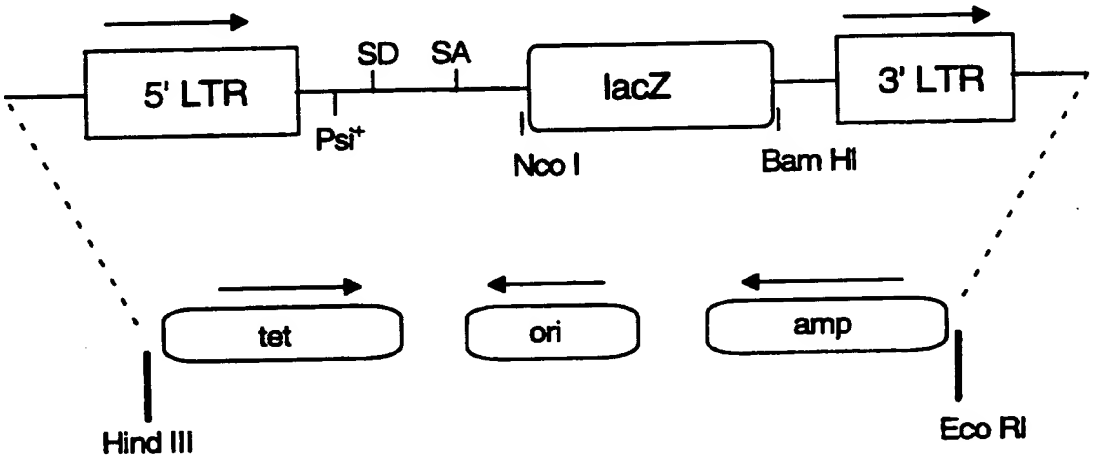
AlwN I 3119

MFG-lacZ



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/11892

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 14829 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 3 September 1992 cited in the application see page 4, line 27 - page 6, line 23 see page 7, line 14 - page 8, line 26 see page 25, line 29 - page 26, line 2 see page 36, line 30 - page 37, line 29	1,3
A	WO,A,88 08450 (FINLAYSON, BIRDWELL ET AL.) 3 November 1988 see page 1, line 1 - line 20 see page 5, line 5 - page 6, line 14 see page 23, line 23 - page 32, line 8; examples 6,7	1-8
A	WO,A,93 18137 (SYSTEMIX, INC.) 16 September 1993 see page 33, line 1 - page 36, line 14	1-8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 January 1996	29. 02. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 14188 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 22 July 1993 see page 15, paragraph 3 - page 22, paragraph 3 ---	1,3
P,X	WO,A,94 29438 (CELL GENESYS, INC.) 22 December 1994 see page 5, line 12 - page 7, line 8 see page 33, line 20 - page 34, line 24; example III -----	1

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/11892

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9214829	03-09-92	AU-B-	663470	12-10-95
		AU-B-	8430291	15-09-92
		EP-A-	0572401	08-12-93
		JP-T-	6504905	09-06-94
WO-A-8808450	03-11-88	NONE		
WO-A-9318137	16-09-93	CA-A-	2131368	16-09-93
		EP-A-	0631618	04-01-95
		JP-T-	7504331	18-05-95
WO-A-9314188	22-07-93	AU-B-	3434393	03-08-93
WO-A-9429438	22-12-94	AU-B-	7246294	03-01-95